Veterinary Parasitology 175 (2011) 360–362

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

Molecular characterization of *Cryptosporidium* in Brazilian sheep

Vagner Ricardo da Silva Fiuza[a,*], Rachel Ingrid Juliboni Cosendey[a], Edwards Frazão-Teixeira[a], Mónica Santín[b], Ronald Fayer[b], Francisco Carlos Rodrigues de Oliveira[a]

[a] *Laboratório de Sanidade Animal, Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Av. Alberto Lamego 2000, Campos dos Goytacazes, Rio de Janeiro 28013-023, Brazil*

[b] *Environmental Microbial and Food Safety Laboratory, Animal and Natural Resources Institute, Agricultural Research Service, United States Department of Agriculture, Building 173, BARC-East, 10300 Baltimore Avenue, Beltsville, MD 20705, USA*

ARTICLE INFO

Article history:
Received 17 May 2010
Received in revised form 13 October 2010
Accepted 14 October 2010

Keywords:
Cryptosporidiosis
PCR
Cryptosporidium ubiquitum

ABSTRACT

Feces were collected from 125 sheep between January and December 2007, on ten farms in the State of Rio de Janeiro, Brazil, and examined for the presence of *Cryptosporidium*. Ninety samples were collected from lambs 2 to 6 months of age, and 35 were from sheep over 12 months of age. All samples were subjected to molecular analysis by polymerase chain reaction (nested PCR) in two steps of the SSU rRNA. Two samples (1.6%) from the lambs were positive, and after sequencing were identified as *Cryptosporidium ubiquitum*. This species has been reported worldwide and it is considered a zoonotic pathogen since it has been found and in several animal species and humans. However, because of the low frequency of *C. ubiquitum* found, the risk for public health in this region may not be high.

In sheep, cryptosporidiosis presents as a mild to severe yellowish liquid diarrhea with a strong odor, loss of weight, depression, abdominal pain, and death usually involving animals up to one month of age (Castro-Hermida et al., 2007; Santín et al., 2007; Geurden et al., 2008; Quílez et al., 2008). *Cryptosporidium* species have been identified in feces of sheep by molecular techniques in the USA, the UK, Italy, Belgium, Spain, Tunisia, China, Australia, and Brazil (Soltane et al., 2007; Geurden et al., 2008; Mueller-Doblies et al., 2008; Quílez et al., 2008; Fayer and Santín, 2009; Féres et al., 2009; Paoletti et al., 2009; Yang et al., 2009; Wang et al., 2010). The following species are mainly responsible for *Cryptosporidium* infections in sheep: *C. parvum*, *C. xiaoi* and *C. ubiquitum*, whereas *C. hominis*, *C. suis*, *C. andersoni*, *C. fayeri* and pig genotype II have already been identified in a very low number of animals (Majewska et al., 2000; Ryan et al., 2005; Ryan et al., 2008; Fayer and Santín, 2009; Fayer et al., 2010). Because sheep can harbor zoonotic species identified in humans with clinical symptoms of cryptosporidiosis (primarily *C. parvum* and *C. hominis*), they should be considered a potential source of infection of *Cryptosporidium* either by direct transmission or by contamination of the environment (Castro-Hermida et al., 2007; Geurden et al., 2008; Paoletti et al., 2009).

Sheep production throughout Brazil is estimated at 15.5 million animals, but is concentrated mainly in the south for meat and wool production and in the northeast for meat and skin production (Viana, 2008). In the State of Rio de Janeiro (RJ), the north area is focused on meat production where the Santa Inês breed, raised in pastures, is the most common (Cosendey et al., 2008a).

Due to the close proximity of sheep to humans and the fact that diarrheic sheep with cryptosporidiosis excrete large quantities of oocysts (Bukhari and Smith, 1997), the present study was conducted to determine the species of *Cryptosporidium* in sheep in the State of Rio de Janeiro and thereby evaluate the risks for human infection.

Fecal samples from sheep were collected in the Northern area of RJ from January to December 2007. Sheep of the Santa Inês breed were selected at random from 10 prop-

* Corresponding author. Tel.: +55 22 2739 7359.
E-mail address: fiuza@uenf.br (V.R.S. Fiuza).

0304-4017
doi:10.1016/j.vetpar.2010.10.036

erties in the municipalities of Carapebus (6), São João da Barra (2), and São Francisco do Itabapoana (2). Samples (15 g) taken directly from the rectum of 125 individual animals were placed in plastic bags. The samples were labeled and packed in insulated containers for transport to the laboratory, where processing was performed within 24 h after collection. All samples were divided into two groups according to age: lambs 2–6 months of age (90 animals) and sheep over 12 months of age (35 animals).

Feces were processed by centrifugation with sucrose (1.1 g/ml) to concentrate and purify oocysts according to Fiuza et al. (2008). The concentration method followed by the nested PCR used in this study has a detection rate of 10, 40 and 80% in samples previously spiked with 10, 100 and 1000 *C. parvum* oocysts per gram of feces, respectively.

For DNA extraction, the DNeasy Tissue Kit (Qiagen®) was used with reagents provided by the manufacturer. Modifications of the protocol included overnight incubation with proteinase K, and elution in 100 μl of AE buffer to increase the quantity of recovered DNA (Santín et al., 2004).

A nested PCR protocol was used to amplify an 830 bp fragment of the SSU rRNA gene from all 125 samples, according to Santín et al. (2004). Before sequencing of the positive samples, the PCR product was purified with two hydrolytic enzymes: Exonuclease I and Shrimp Alkaline Phosphatase, in a specially formulated buffer (ExoSAP-IT, USB Corporation). After purification, the product was sequenced in both directions using the same PCR primers used for the second amplification in 10 μl reactions, Big Dye Chemistries, in an ABI 3100 sequencer analyzer (Applied Biosystems). The sequences of each strand were aligned and examined with Lasergene software (DNASTAR), and submitted to the Basic Local Alignment Search Tool (BLAST) analysis to identify similarities with the GenBank sequences (Altschul et al., 1997).

Samples (1.6%), from 2 lambs less than 6 months of age from Carapebus, were positive for *Cryptosporidium* and after sequencing and comparison with the GenBank database, homology was observed with *C. ubiquitum* (previously known as cervine genotype). Both nucleotide sequences were identical and can be accessed through GenBank under access number HM772993.

In an epidemiological study of cryptosporidiosis, it is of fundamental importance to identify the species observed because it is the only way to evaluate contamination risks to other animal species and humans. Due to the great morphological similarity among species of *Cryptosporidium*, microscopic observation of oocysts alone is insufficient for identification of species which requires molecular data (Fayer and Xiao, 2007). Without molecular data microscopic detection does not provide relevant information on the zoonotic potential of the observed oocysts or enable evaluation of the risk of infection to other animals in the vicinity (Fayer and Santín, 2009).

Currently, *C. ubiquitum* has been reported in a wide variety of hosts (Ong et al., 2002; Xiao et al., 2002; da Silva et al., 2003; Ryan et al., 2003), but appears most prevalent in lambs (Ryan et al., 2005; Santín et al., 2007). Importantly, the diagnosis of this species must be interpreted with caution when *Rsa*I restriction enzymes are used for the COWP gene amplification because *C. ubiquitum* and *C. hominis* have the same restriction site (Ong et al., 2002; Santín and Fayer, 2007).

In a study conducted in Australia, 447 fecal samples from pre-weaned sheep up to eight weeks of age were analyzed by nested PCR of the 18S rRNA followed by sequencing of positive samples (Yang et al., 2009). *Cryptosporidium ubiquitum* was observed in 2.2% of the total samples (10/447), which is similar to the prevalence described in Brazil by this study [1.6% (2/125)]. Geurden et al. (2008) found a slightly higher prevalence in a survey conducted in ten properties in Belgium, where 6.5% (9/137) of fecal samples from lambs up to 10 weeks of age were positive for *C. ubiquitum*. A study in the USA by Santín et al. (2007), in which fecal samples were collected at 7, 14 and 21 days of age from 32 lambs and examined by nested PCR, showed a high prevalence of *C. ubiquitum* in lambs less than a month old. Of these, 22 were eliminating *C. ubiquitum* and three different sequences of *C. ubiquitum* (cervine 1–3) were observed. The sequences obtained in the present study (HM772993) have 99.8% homology (656/657) with the cervine 2 genotype described by Santín et al. (2007) (EF362480) in six of the 22 animals. The same sequence of cervine genotype 2 has been reported in the United Kingdom and was previously identified as a novel genotype (Elwin and Chalmers, 2008).

In Brazil, diagnostics based on microscopy in sheep feces have shown prevalence rates ranging between 3.7 and 47.0% (Green et al., 2004; Tembue et al., 2006; Cosendey et al., 2008a; Cosendey et al., 2008b). Féres et al. (2009) collected 460 fecal samples from 21 sheep farms in the State of Sao Paulo (SP). After screening with malachite green, 31 positive samples were analyzed by PCR of the 18S rRNA. After sequencing was performed on one sample from each property in the study, three samples were successfully sequenced: *C. parvum* type A, *C. parvum* type B, and *C. ubiquitum*. Because GenBank access numbers for these sequences were not provided we could not compare those sequences with sequences found in the present study. Also, in SP (Paz e Silva, 2007), 100 samples from sheep of different ages were collected and analyzed by RFLP PCR. Two samples presented restriction patterns compatible with *C. parvum*, two with *C. bovis* and 19 compatible with *C. felis* or *C. ubiquitum*. According to information provided by the authors themselves, both *C. felis* and *C. ubiquitum* have the same restriction sites; therefore, they could not conclude if it was *C. felis* or *C. ubiquitum* because sequencing of the positive PCR samples was not performed.

The first reports of *C. ubiquitum* in humans were found by Ong et al. (2002) in fecal samples from patients with clinical symptoms consistent with cryptosporidiosis and by Trotz-Williams et al. (2006) in a fecal sample after PCR amplification of the 18S rRNA. Subsequently, sporadic cases of this species affecting humans have been described (Feltus et al., 2006; Leoni et al., 2006; Soba et al., 2006) and therefore *C. ubiquitum* should be considered a potential emerging zoonotic pathogen (Santin and Fayer, 2007).

In the present study *C. ubiquitum* was observed only in lambs. In the U.S. and Belgium, this species is most prevalent in lambs when compared with weaned and adults (Santín et al., 2007; Geurden et al., 2008). However, in Australia, *C. ubiquitum* was more prevalent in weaned ani-

mals and was the most prevalent among the eight species and genotypes that were diagnosed (Ryan et al., 2005).

It is important to consider that the low frequency of *Cryptosporidium* in this study may be related to the number of samples collected per animal, which was performed on only one occasion. Studies have shown that animals negative for oocysts in one sample analysis may be positive in other samples collected from the same animal within an interval of a few days (Santín et al., 2007). The age of sheep is also another factor to be considered. In this animal species, cryptosporidiosis also appears to have a higher prevalence in lambs less than one month old, which is similar to results observed by Santín et al. (2007), Castro-Hermida et al. (2007) and Quílez et al. (2008).

The results of this study demonstrate the need for further comprehensive molecular studies of sheep cryptosporidiosis in Brazil, with an epidemiological design and sample size determination based on the number of animals per region. Because of the low frequency of *C. ubiquitum* found, the risk for public health in this region may not be high. Feces from humans who live in the same area should also be examined for a conclusive study.

Acknowledgements

The authors would like to thank Samira Salim Mello Gallo for technical support, and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Bukhari, Z., Smith, H.V., 1997. *Cryptosporidium parvum*: oocyst excretion and viability patterns in experimentally infected lambs. Epidemiol. Infect. 119, 105–108.

Castro-Hermida, J.A., González-Warleta, M., Mezo, M., 2007. Natural infection by *Cryptosporidium parvum* and *Giardia duodenalis* in sheep and goats in Galacia (NW Spain). Small Rumin. Res. 72, 96–100.

Cosendey, R.I.J., Fiuza, V.R.S., Teixeira, C.S., Oliveira, F.C.R., 2008a. Freqüência de oocistos de coccídios do gênero *Cryptosporidium* em ovinos no estado do Rio de Janeiro. Rev. Bras. Saúde Prod. An. 9, 687–695.

Cosendey, R.I.J., Fiuza, V.R.S., Oliveira, F.C.R., 2008b. Importância do manejo na criptosporidiose em criações de ovinos. Rev. Bras. Parasitol. Vet. 17, 209–214.

da Silva, A.J., Cacciò, S., Williams, C., Won, K.Y., Nace, E.K., Whittier, C., Pieniazek, N.J., Eberhard, M.L., 2003. Molecular and morphologic characterization of a *Cryptosporidium* genotype identified in lemurs. Vet. Parasitol. 111, 297–307.

Elwin, K., Chalmers, R.M., 2008. Contemporary identification of previously reported novel *Cryptosporidium* isolates reveals *Cryptosporidium bovis* and the cervine genotype in sheep (*Ovis aries*). Parasitol. Res. 102, 1103–1105.

Fayer, R., Santín, M., 2009. *Cryptosporidium xiaoi* n. sp. (Apicomplexa: Cryptosporidiidae) in sheep (*Ovis aries*). Vet. Parasitol. 164, 192–200.

Fayer, R., Xiao, L., 2007. Cryptosporidium and Cryptosporidiosis, 2nd ed. CRC Press, Boca Raton.

Fayer, R., Santín, M., Macarisin, D., 2010. *Cryptosporidium ubiquitum* n. sp. in animals and humans. Vet. Parasitol. 172, 23–32.

Feltus, D.C., Giddings, C.W., Schneck, B.L., Monson, T., Warshauer, D., McEvoy, J.M., 2006. Evidence supporting zoonotic transmission of *Cryptosporidium* spp. in Wisconsin. J. Clin. Microbiol. 44, 4303–4308.

Féres, F.C., Lombardi, A.L., Carvalho, M.P.P., Mendes, L.C.N., Peiró, J.R., Cadioli, F.A., Meireles, M.V., Perri, S.H.V., Feitosa, F.L.F., 2009. Ocorrência e caracterização molecular de *Cryptosporidium* em cordeiros. Arq. Bras. Med. Vet. Zootec. 61, 1002–1005.

Fiuza, V.R.S., Cosendey, R.I.J., Oliveira, F.C.R., 2008. Criptosporidiose suína associada aos sistemas de produção no Estado do Rio de Janeiro. Rev. Bras. Parasitol. Vet. 17, 224–229.

Geurden, T., Thomas, P., Casaert, S., Vercruysse, J., Claerebout, E., 2008. Prevalence and molecular characterisation of *Cryptosporidium* and *Giardia* in lambs and goat kids in Belgium. Vet. Parasitol. 155, 142–145.

Green, R.E., Amarante, A.F.T., Mascarini, L.M., 2004. The seasonal distribution of *Cryptosporidium oocysts* in sheep raised in the State of São Paulo. Rev. Bras. Parasitol. Vet. 13, 125–127.

Leoni, F., Amar, C., Nichols, G., Pedraza-Diaz, S., McLauchlin, J., 2006. Genetic analysis of *Cryptosporidium* from 2414 humans with diarrhoea in England between 1985 and 2000. J. Med. Microbiol. 55, 703–707.

Majewska, A.C., Werner, A., Sulima, P., Luty, T., 2000. Prevalence of *Cryptosporidium* in sheep and goats bred on five farms in west-central region of Poland. Vet. Parasitol. 89, 269–275.

Mueller-Doblies, D., Giles, M., Elwin, K., Smith, R.P., Clifton-Hadley, F.A., Chalmers, R.M., 2008. Distribution of *Cryptosporidium* species in sheep in the UK. Vet. Parasitol. 154, 214–219.

Ong, C.S., Eisler, D.L., Alikhani, A., Fung, V.W., Tomblin, J., Bowie, W.R., Isaac-Renton, J.L., 2002. Novel *Cryptosporidium* genotypes in sporadic cryptosporidiosis cases: first report of human infections with a cervine genotype. Emerg. Infect. Dis. 8, 263–268.

Paoletti, B., Giangaspero, A., Gatti, A., Iorio, R., Cembalo, D., Milillo, P., Traversa, D., 2009. Immunoenzymatic analysis and genetic detection of *Cryptosporidium parvum* in lambs from Italy. Exp. Parasitol. 122, 349–352.

Paz e Silva, F.M., 2007. Diagnóstico e caracterização molecular de Giardia duodenalis e *Cryptosporidium* spp. em amostras fecais de bovinos e ovinos. Master of Parasitic Diseases of Animals Thesis. Botucatu, SP, Brazil.

Quílez, J., Torres, E., Chalmers, R.M., Hadfield, S.J., del Cacho, E., Sánchez-Acedo, C., 2008. *Cryptosporidium* genotypes and subtypes in lambs and goat kids in Spain. Appl. Environ. Microbiol. 74, 6026–6031.

Ryan, U.M., Xiao, L., Read, C., Zhou, L., Lal, A.A., Pavlasek, I., 2003. Identification of novel *Cryptosporidium* genotypes from the Czech Republic. Appl. Environ. Microbiol. 69, 4302–4307.

Ryan, U.M., Bath, C., Robertson, I., Read, C., Elliot, A., Mcinnes, L., Traub, R., Besier, B., 2005. Sheep may not be an important zoonotic reservoir for *Cryptosporidium* and *Giardia* parasites. Appl. Environ. Microbiol. 71, 4992–4997.

Ryan, U.M., Power, M., Xiao, L., 2008. *Cryptosporidium fayeri* n. sp. (Apicomplexa: Cryptosporidiidae) from the Red Kangaroo (*Macropus rufus*). J. Eukaryot. Microbiol. 55, 22–26.

Santín, M., Fayer, R., 2007. Intragenotypic variations in the *Cryptosporidium* sp. cervine genotype from sheep with implications for public health. J. Parasitol. 93, 668–672.

Santín, M., Trout, J.M., Xiao, L., Zhou, L., Greiner, E., Fayer, R., 2004. Prevalence and age-related variation of *Cryptosporidium* species and genotypes in dairy calves. Vet. Parasitol. 122, 103–117.

Santín, M., Trout, J.M., Fayer, R., 2007. Prevalence and molecular characterization of *Cryptosporidium* and *Giardia* species and genotypes in sheep in Maryland. Vet. Parasitol. 146, 17–24.

Soba, B., Petrovec, M., Mioc, V., Logar, J., 2006. Molecular characterization of *Cryptosporidium* isolates from humans in Slovenia. Clin. Microbiol. Infect. 12, 918–921.

Soltane, R., Guyot, K., Dei-Cas, E., Ayadi, A., 2007. Prevalence of *Cryptosporidium* spp. (Eucoccidiorida: Cryptosporiidae) in seven species of farm animals in Tunisia. Parasite 14, 335–338.

Tembue, A.A.M., Alves, L.C., Borges, J.C., Faustino, M.A.G., Machado, E.C.L., 2006. Ocorrência de *Cryptosporidium* spp. em ovinos no Município de Ibimirim. Estado de Pernambuco. Ciênc. Vet. Tróp. Recife-PE 9, 41–43.

Trotz-Williams, L.A., Martin, D.S., Gatei, W., Cama, V., Peregrine, A.S., Martin, S.W., Nydam, D.V., Jamieson, F., Xiao, L., 2006. Genotype and subtype analyses of *Cryptosporidium* isolates from dairy calves and humans in Ontario. Parasitol. Res. 99, 346–352.

Viana, J.G.A., 2008. Panorama geral da ovinocultura no mundo e no Brasil. Rev. Ovinos. 4, 44–47.

Wang, Y., Feng, Y., Cui, B., Jian, F., Ning, C., Wang, R., Zhang, L., Xiao, L., 2010. Cervine genotype is the major *Cryptosporidium* genotype in sheep in China. Parasitol. Res. 106, 341–347.

Xiao, L., Sulaiman, I.M., Ryan, U.M., Zhou, L., Atwill, E.R., Tischler, M.L., Zhang, X., Fayer, R., Lal, A.A., 2002. Host adaptation and host–parasite co-evolution in *Cryptosporidium*: implications for taxonomy and public health. Int. J. Parasitol. 32, 1773–1785.

Yang, R., Jacobson, C., Gordon, C., Ryan, U., 2009. Prevalence and molecular characterization of *Cryptosporidium* and *Giardia* species in pre-weaned sheep in Australia. Vet. Parasitol. 161, 19–24.